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The positive role of the carboxyl terminus of the γ subunit of retinal cGMP-phosphodiesterase in maintaining phosphodiesterase activity in vivo ☆

Stephen H. Tsang [a,1], Clyde K. Yamashita [a,1], Won-Ho Lee [a], Chyuan-Sheng Lin [c], Stephen P. Goff [c], Peter Gouras [d], Debora B. Farber [a,b,*]

[a] *Jules Stein Eye Institute, UCLA School of Medicine, 100 Stein Plaza, Los Angeles, CA 90095-7000, USA*
[b] *Molecular Biology Institute, UCLA School of Medicine, 100 Stein Plaza, Los Angeles, CA 90095-7000, USA*
[c] *Department of Biochemistry and Molecular Biophysics, Howard Hughes Medical Institute, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA*
[d] *Department of Ophthalmology, Edward S.Harkness Eye Institute, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA*

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Abstract

The inhibitory rod cyclic GMP-phosphodiesterase γ subunit, PDEγ, is a key component of the photoresponse and is required to support rod integrity. $Pdeg^{tm1}/Pdeg^{tm1}$ mice that lack PDEγ due to a targeted disruption of the gene encoding PDEγ, (*Pdeg*) suffer from a very rapid and severe photoreceptor degeneration. Previously, deletions in the carboxyl-terminal domain of PDEγ blocked its ability to inhibit trypsin-activated PDE activity, in vitro. In other words, these mutations eliminated PDEγ's control on the catalytic activity of PDEα and PDEβ. To study the in vivo effects resulting from the deletion of the last seven amino acids of the PDEγ carboxyl terminal, this PDEγ allele (*Del7C*) was introduced as a transgene $Pdeg^{tm1}/Pdeg^{tm1}$ mice. These animals could only synthesize transgenic mutant PDEγ. The mutant retinas were expected to display a higher basal level of PDE activity and lower cGMP levels in light and darkness than the PDEγ knockout mice, which would allow the rescue of their photoreceptors. Instead, our results showed that the *Del7C* transgene could not complement the $Pdeg^{tm1}/Pdeg^{tm1}$ mutant for photoreceptor survival. In fact, animals carrying the *Del7C* transgene have low PDE activity as well as reduced PDEα and PDEβ content.

Keywords: Transgenic animals; Knockout animals; Photoreceptor transduction; Retinal degeneration; Phosphodiesterase

Abbreviations: PDE, cGMP phosphodiesterase; *Pdeg*, gene encoding the γ subunit of the cGMP phosphodiesterase; Tα, transducin alpha; $Pdeg^{tm1}/Pdeg^{tm1}$, a mouse line carrying a targeted disruption of *Pdeg*; Tg*Del7C*, transgenic mice that only express a mutant γ subunit of PDE; ERG, electroretinogram; ROS, rod outer segments

1. Introduction

Neurotransmitters and hormones signal their target cells through G-protein-coupled receptors. The visual response to a single photon (Stryer, 1991; Yarfitz & Hurley, 1994) starts with the activation of rhodopsin, which causes transducin (a heterotrimeric G-protein {Tαβγ}) (Fung, Hurley, & Stryer, 1981) to exchange its bound guanosine diphosphate for guanosine triphosphate (GTP). Activated transducin molecules, Tα-GTP, then dissociate from Tβγ molecules and bind to the inhibitory γ subunits of cGMP-phosphodiesterase (PDE, a heterotetrameric enzyme, $PDE\alpha\beta\gamma_2$) (Baehr, Devlin, & Applebury, 1979; Fung, Young, Yamane,

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* Corresponding author. Tel.: +1-310-206-7375; fax: +1-310-794-7904.
E-mail address: farber@jsei.ucla.edu (D.B. Farber).

[1] These authors contributed equally to the work.

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& Griswold-Prenner, 1990), thereby relieving the inhibition that the two PDEγ subunits exert on the catalytic PDEα- and β- subunits (Baehr et al., 1979; Deterre, Bigay, Forquet, Robert, & Charbre, 1988; Fung et al., 1981; Fung et al., 1990; Wensel & Stryer, 1986). The resulting activation of PDEαβ lowers the levels of cGMP (Miki, Baraban, Keirns, Boyce, & Bitensky, 1975) which leads to closure of cGMP-gated Na^+/Ca^{2+} channels on the plasma membrane. This reduces the entry of Na^+ and Ca^{2+} into the cytoplasm, causing the entire rod to hyperpolarize.

The regions of PDEγ that are required for its various functions have been studied in the last decade using reconstituted systems. PDEγ contains a central lysine-rich region, spanning residues Arg24 to Gly45, in which 10 of 13 amino acids are basic. These residues contain one site for interaction with Tα (Lipkin, Dumler, Muradov, Artemyev, & Etingof, 1988), and are essential for binding to the PDE catalytic core (Artemyev & Hamm, 1992). The region involved in inhibiting PDE catalytic activity is thought to lie at the carboxyl terminus, and is different from the region required for binding Tα and the catalytic PDE subunits (Lipkin et al., 1988). In fact, deletions and point mutations in the carboxyl terminus were shown not to affect the binding of PDEγ to PDEαβ, but to decrease inhibition of PDE activity (Berger, Cerione, & Erickson, 1997; Gonzalez, Cunnick, & Takemoto, 1991; Takemoto, Hurt, Oppert, & Cunnick, 1992). Further, peptides corresponding to the carboxyl terminus were found to inhibit trypsin-activated PDE (Granovsky, Natochin, & Artemyev, 1997).

To examine the in vivo function of the inhibitory γ subunits, we generated a mouse line, $Pdeg^{tm1}/Pdeg^{tm1}$, carrying a disruption of the PDEγ gene (Tsang et al., 1996). Based on previous in vitro studies, the loss of the inhibitory PDEγ subunit was expected to generate a constitutively active PDEαβ core, as in light activation. However, the data surprisingly showed that the loss of the inhibitory PDEγ subunit prevented the functioning of PDE and elevated rather than depressed cGMP levels in the developing photoreceptors of homozygous knockout mice. Examination of mutant retinal extracts showed that the PDEαβ catalytic dimer was formed but lacked hydrolytic activity. These results suggested that an interaction between the inhibitory PDEγ subunit and the PDE catalytic core may be critical for the proper action of the enzyme, as well as for the proper folding or conformation of the catalytic sites of the PDEαβ core. The positive participation of PDEγ in the formation of an active PDE complex was unexpected.

To test whether the PDEγ domain that maintains the PDE catalytic core function is in the carboxyl terminus in vivo, various mutant and wild-type PDEγ cDNAs under the control of the opsin promoter were constructed and used to generate transgenic mice by conventional means (Hogan, Beddington, Costantini, & Lacy, 1994). The transgenes were introduced into homozygous PDEγ knockout mice by breeding, and animals of the appropriate genotype were identified by PCR and Southern blot analysis of genomic DNA. In this paper, we examine the physiological effects of deletion of the last seven amino acids (*Del7C*) previously shown to decrease the inhibitory potential of the PDEγ subunit (Gonzalez et al., 1991; Lipkin et al., 1990). This mutant transgene would be expected to result in the formation of a constitutively active PDE and thus to produce low levels of cGMP in the animals carrying it. The retinas of these mice would also be expected to be in a state of constant light adaptation and have a saturated rod response. Contrary to these expectations, we found that the mutant retinas have a loss of PDE activity and a decreased PDEα and PDEβ content. Furthermore, they have minimal electroretinogram (ERG) responses.

2. Materials and methods

2.1. Generation of mutant mouse lines

The mice studied in these experiments were used in accordance to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. DNA constructs for the expression of PDEγ contained 4.4 kb of the mouse opsin promoter, the complete open reading frame of the PDEγ cDNA (Tuteja & Farber, 1988), and the polyadenylation signal of the mouse protamine gene (Lem, Applebury, Falk, Flannery, & Simon, 1991). The *Del7C* mutation was introduced by a standard PCR-based site-specific mutagenesis strategy (Tsang et al., 1998). The entire PDEγ cDNA coding region in the transgenic construct was sequenced to confirm the introduction of the deletion and that no other changes had been created inadvertently. *Kpnl* and *Xbal* were used to excise vector sequences from the constructs. Oocytes were obtained from superovulated F1(DBA × C57BL6) females mated with homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ males. The construct was injected into the male pronuclei of oocytes under a depression slide chamber. These microinjected oocytes were cultured overnight in M16 and transferred into the oviducts of 0.5-day p.c. pseudopregnant F1 females. The resulting transgenic mice were then backcrossed to $Pdeg^{tm1}/Pdeg^{tm1}$ mice to place the transgene into the knockout background. The mice were also tested for the absence of the *rd1* mutation (Pittler & Baehr, 1991).

2.2. Identification of transgenic mice

DNA was isolated from tail tips or liver samples by homogenizing the tissue, digesting extensively with proteinase K and extracting with phenol. DNAs were analyzed by PCR. The DNAs were also digested by SacI

and analyzed by Southern blot hybridization with a PDEγ cDNA probe. Additional restriction digests were performed to analyze the structure of the integrated sequences, and to insure that the DNA flanking the transgene was intact.

2.3. Immunoblot analyses

Proteins were separated by polyacrylamide gel electrophoresis on either a 12% acrylamide/1.5% bis-acrylamide (for the PDEγ subunit) or a 6.5% acrylamide/1.5% bis-acrylamide (for PDEα and β subunits) in a tris/tricine buffer system. Proteins were then transferred to 0.2 μm Immuno-blot PVDF membranes (BioRad Laboratories, Hercules, CA) by the method of Towbin, Staehelin, and Gordon (1979), overnight, at 4 $V\,cm^{-1}$. Membranes were blocked in 3% bovine serum albumin (BSA) in 500 mM NaCl, 20 mM Tris, pH 7.6, and 0.1% Tween 20 (BSA-TTBS). For the detection of PDEγ, blots were incubated with a 1:1000 dilution of a polyclonal peptide antiserum directed against the *N*-terminal (amino acid residues 2–16) of the PDEγ subunit. The PDEα and β subunits were detected by incubation with a 1:2000 dilution of a polyclonal antiserum raised against the native enzyme (both antisera were the generous gift of Dr. Bernard K.K. Fung). Western blots were visualized with the DuoLux Chemiluminescence substrate kit (Vector Laboratories, Inc., Burlingame, CA) utilizing a goat-anti-rabbit IgG-alkaline phosphatase conjugate. Blots were exposed to Hyperfilm-MP (Amersham Pharmacia Biotech, Piscataway, NJ) preflashed to increase sensitivity and linearity according to the Sensitize™ protocol (Amersham Pharmacia Biotech). Signals were analyzed and quantified on a Power-Macintosh 9600 computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.4. Histology

Mice were euthanized with an intraperitoneal injection of ketamine. Each eye was rapidly removed, punctured at 12:00 along the limbus, and placed in a separate solution of 3% glutaraldehyde in phosphate buffered saline. After fixation for 1–2 days, the eyes were washed with saline and the 12:00 limbal puncture was used to orient the right and left eyes, which were kept in separate buffer, so that the posterior segment containing the retina could be sectioned along the vertical meridian. A rectangular piece containing the entire retina from superior to inferior ora serrata, including the optic nerve was prepared for postfixing in osmic acid, dehydration, and epon embedding. A corner was cut out at the superior ora to allow identification of the upper retinal half of the segment. Sectioning proceeded along the long axis of the segment so that each section contained both upper and lower retina as well as the posterior pole. These segments were sectioned semi-serially, stained with toluidine blue, mounted, and examined by light microscopy.

2.5. Electroretinography

ERGs were obtained from anesthetized animals using a saline moistened cotton wick electrode that contacted the cornea. Mice were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) administered either intraperitoneally or intramuscularly. The pupils were dilated with 1% phenylephrine HCl and 1% cyclopentolate. Body temperature was maintained at 37 °C by a heated stage on which the mouse was laid. A 30 gauge needle was placed subcutaneously on the forehead as a reference electrode and a ground electrode subcutaneously on the trunk. The stimuli were light flashes obtained from a Grass Instruments Inc. stroboscope removed from its housing and mounted in a metal box with a circular aperture, 3 cm in diameter, placed 9 cm from the center of the pupil of the mouse. Neutral density and/or spectral filters were placed between this aperture and the cornea to vary the energy and wavelength of the flash. Responses were detected with a Nicolet Instruments CA-1000 oscilloscope, averaging from 3 to 20 responses to the same flash intensity. Mice were dark adapted for at least 6 h before testing. Stimulation was begun with 4.8 log units of neutral density filtering before the strobe flash and responses were averaged to one flash every 3 s. At high flash intensities, each flash was presented every 15 s. (The duration of a flash is nominally 10 μs. The frequency response of the recording system extends from 1 to 500 Hz.)

2.6. PDE activity assay

To measure basal PDE activity, animals were dark-adapted overnight and retinas dissected under far red light (Kodak Type 11 filter), and homogenized in hypotonic buffer (10 mM Tris, pH 7.6, 2 mM EDTA, 2 mM EGTA, 2 μg/ml leupeptin, and 2 μg/ml pepstatin). Homogenates were centrifuged for 20 min at $12{,}000 \times g$ and supernatants collected. Aliquots were taken for protein determination by the method of Peterson (1977) using BSA as standard, and for cGMP-PDE activity measurement by the method of Farber and Lolley (1976), using 250 μM cGMP as substrate. Each reaction was carried out in triplicate. Three to 10 μg of retinal protein (depending on the sample) were incubated for up to 10 min at 37 °C with the substrate in 40 mM Tris buffer, pH 7.6, containing 200,000 cpm ^{3}H-cGMP, 5 mM $MgCl_2$, and 1 mM DTT. The reaction was terminated by heating to 80 °C for 3 min. 0.4 U of calf intestinal alkaline phosphatase was then added to the

sample and incubation proceeded at 37 °C for 10 min. The resulting ^{3}H-guanosine was separated from other nucleotides by a resin slurry (AG1-X2, 50–100 mesh, Biorad Laboratories) and radioactivity was quantified in a scintillation counter. Results were expressed as nmol of cGMP hydrolyzed min^{-1} mg^{-1} total protein. To measure trypsin-stimulated PDE activity, trypsin (3u) was added to a 20 ml sample. After 3 min incubation at room temperature, the reaction was terminated with 200 mg soybean trypsin inhibitor.

3. Results

3.1. The Del7C mutant transgene temporarily restores the photoreceptors of the PDEγ knockout mice

The *Del7C* mutation in the C-terminus of PDEγ has been shown to block the inhibition of the catalytic core PDEαβ that is mediated by the wild-type PDEγ in vitro (Gonzalez et al., 1991; Lipkin et al., 1990). To test the effects of this carboxyl-terminal mutation on PDE function in vivo, transgenic lines (Tg) expressing the *Del7C* mutant allele were generated and crossed with $Pdeg^{tm1}/Pdeg^{tm1}$ to obtain mice that only expressed the mutant PDEγ. Two independent Tg*Del7C* lines (1 and 2) were used for this study. As controls, eight lines of transgenic mice expressing the wild-type allele in the same background were also developed (Tsang et al., 1998).

As expected, the wild-type transgene rescued the $Pdeg^{tm1}/Pdeg^{tm1}$ mice photoreceptors. However, the degeneration of the parental knockout mice was only slowed down a few days by the mutant *Del7C* transgene. Fig. 1 shows light micrographs of retinal sections obtained from $Pdeg^{tm1}/Pdeg^{tm1}$ mice with and without the *Del7C* transgene at different times during postnatal development. At 14 days of age, retinas from both $Pdeg^{tm1}/Pdeg^{tm1}$ mice with the wild-type control transgene (A) and with the *Del7C* transgene (B) showed 10–12 rows of photoreceptor nuclei. In contrast, the parental $Pdeg^{tm1}/Pdeg^{tm1}$ mice without the *Del7C* transgene showed two to three rows of outer nuclear layer (C). At 17 days of age, the number of photoreceptor nuclei had decreased to five rows in $Pdeg^{tm1}/Pdeg^{tm1}$ mice with the *Del7C* transgene (D) and one to two rows of nuclei in the parental $Pdeg^{tm1}/Pdeg^{tm1}$ mice without the *Del7C* (E). By the 21st postnatal day, the $Pdeg^{tm1}/Pdeg^{tm1}$ mice with the *Del7C* transgene (F) looked like the parental $Pdeg^{tm1}/Pdeg^{tm1}$ mice without the transgene at day 17 (E). These knockout mice had very few photoreceptor nuclei left at 21 days of age (G). A few days later retinas from both PDEγ knockout mice with or without the *Del7C* transgene showed comparable photoreceptor-less morphology. Thus, the time course of degeneration of *Del7C* mice is slower than that of the $Pdeg^{tm1}/Pdeg^{tm1}$ mice by approximately a week.

3.2. Physiological features of the mutant mice

To assess the photoresponses at a time when photoreceptors were still present in large numbers, ERG measurements were made in 12-day-old animals. The amplitude of the b-waves in the ERG of the two independent homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ lines (−/−) with the *Del7C* transgene (1 and 2) was larger and the sensitivity higher than those of the $Pdeg^{tm1}/Pdeg^{tm1}$ mice (−/−) at high light intensities (Fig. 2). However, these responses were much smaller than those of wild-type mice and decayed gradually with increasing age, indicating that the abnormality was progressive (Fig. 3).

3.3. Biochemical analyses of mutant mice

Immunoblots of proteins from retinal homogenates of 12-day-old postnatal control, $Pdeg^{tm1}/Pdeg^{tm1}$ knockout, and both lines of Tg*Del7C* mice (Fig. 4A) revealed the absence of the PDEγ subunit in the knockout (lane 2) and Tg*Del7C* animals (lanes 3 and 4). Therefore, we considered both Tg*Del7C* lines indistinguishable and equivalent and pooled retinas of these mice in further experiments. Surprisingly, expression of the catalytic PDEα and β subunits was reduced by approximately 90% in the Tg*Del7C* animals when compared to both the +/+ control and $Pdeg^{tm1}/Pdeg^{tm1}$ knockout animals (Fig. 4B). This reduction in the PDE catalytic core expression of the Tg*Del7C* animals was reflected in the lower basal PDE activity of the dark-adapted Tg*Del7C* retinas when compared to that of +/+ control retinas (18.3 ± 1.3 and 40.0 ± 9.0 nmol min^{-1} mg^{-1} total protein, respectively). Furthermore, measurement of total PDE activity by trypsin activation showed that the retinal Tg*Del7C* PDE activity was only 2.3% of that of the +/+ control retina (162 nmol min^{-1} mg^{-1} total protein in the Tg*Del7C* compared to 7078 nmol min^{-1} mg^{-1} total protein in the +/+ control).

4. Discussion

During light activation, Tα binds to PDEγ inducing a displacement of PDEγ's carboxyl terminus from the core PDEαβ catalytic sites and promoting cGMP hydrolysis (Skiba, Artemyev, & Hamm, 1995). A conformational change at this carboxyl terminus may in turn make PDEγ's central region more accessible to Tα binding (Skiba et al., 1995), leading to acceleration of Tα-GTPase activity and deactivation of the photoresponse.

In vitro experiments have shown that mutations in the carboxyl terminus of PDEγ block its ability to inhibit trypsin-activated PDE activity (Artemyev, Natochin, Busman, Schey, & Hamm, 1996a; Artemyev,

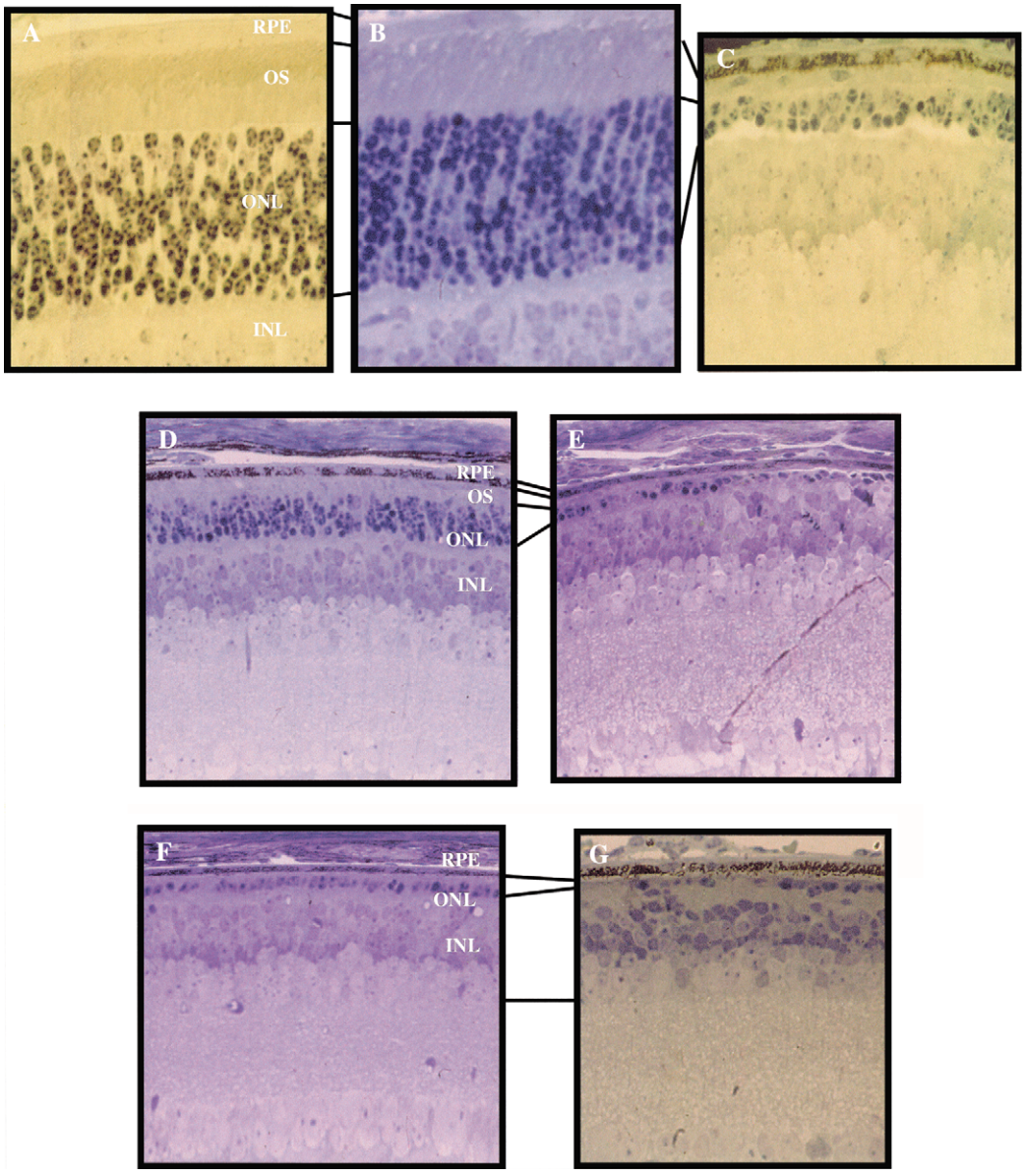

Fig. 1. Light micrographs of retinas from: (A) an adult $Pdeg^{tm1}/Pdeg^{tm1}$ mouse with a wild-type transgene, (B) a 14-day-old $Pdeg^{tm1}/Pdeg^{tm1}$ mouse with the *Del7C* transgene, (C) a 14-day-old $Pdeg^{tm1}/Pdeg^{tm1}$ mouse, (D) and (E) a 17-day-old $Pdeg^{tm1}/Pdeg^{tm1}$ mouse with the *Del7C* transgene and a $Pdeg^{tm1}/Pdeg^{tm1}$ mouse, respectively, (F) and (G) a 21-day-old $Pdeg^{tm1}/Pdeg^{tm1}$ mouse with the *Del7C* transgene and a $Pdeg^{tm1}/Pdeg^{tm1}$ knockout mouse, respectively. RPE: retinal pigment epithelium; OS: outer segments; ONL: outer nuclear layer; INL: inner nuclear layer.

Surendran, Lee, & Hamm, 1996b; Berger, Cerione, & Erickson, 1999; Gonzalez et al., 1991; Stryer, 1991; Takemoto et al., 1992). The inhibitory domain of PDEγ is located within the last 11 amino acids at the carboxyl terminus (Skiba et al., 1995). A carboxyl-terminal PDEγ peptide corresponding to amino acid residues 68–87 alone is sufficient to completely inhibit trypsin-activated PDE (Skiba et al., 1995). Brown (1992) also have shown that carboxyl residues 74–87 are essential for inhibiting but not for binding to the PDEαβ catalytic core. Interestingly, recombinant PDEγ truncated at amino acid residue 74 stimulated rather than inhibited trypsin-activated PDE activity (Brown, 1992). All these observations indicate that in vitro, the PDEγ carboxyl tail is necessary and sufficient to inhibit the PDEαβ catalytic activity (Gonzalez et al., 1991; Lipkin et al., 1993; Natochin & Artemyev, 1996; Skiba et al., 1995; Takemoto et al., 1992). In addition, *Del7C* was found to decrease PDEγ inhibitory properties without a loss of its affinity for the catalytic core (Natochin et al., 1991). Based on these studies, we hypothesized that in the dark the *Del7C* retina should display a higher basal level of PDE activity.

Surprisingly, the PDE activity in retinas of dark-adapted Tg*Del7C* 12-day-old mice, was higher than that in retinas of age-matched $Pdeg^{tm1}/Pdeg^{tm1}$ animals, but

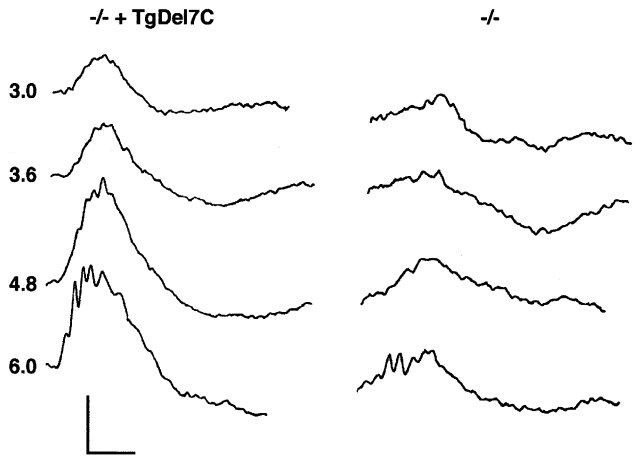

Fig. 2. The corneal ERGs to white flashes of different intensities depending on the amount of neutral density filtering placed before the flash. The numbers on the left indicate the log relative energy of the light stimulus. The left column of responses are from the homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ mice (−/−) with the *Del7C* transgene and the right from $Pdeg^{tm1}/Pdeg^{tm1}$ mice (−/−). The calibration at the lower left indicates 50 μV vertically and 50 ms horizontally.

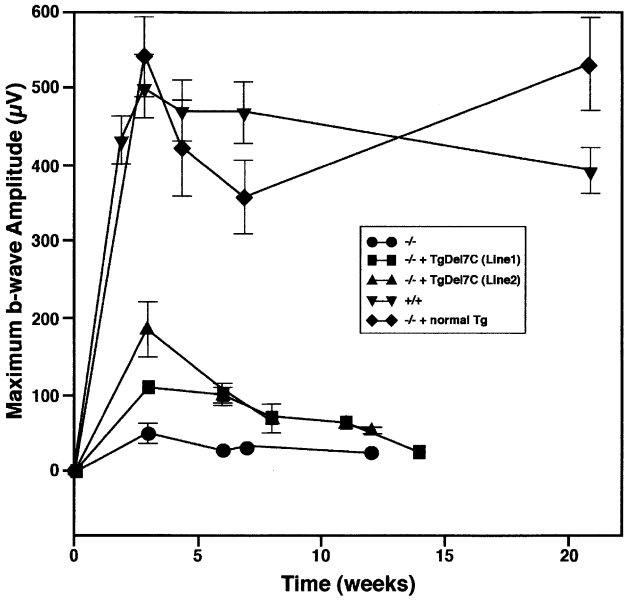

Fig. 3. The relationship between the maximum ERG b-wave amplitude and the age of the mouse in weeks. Each point represents the average response of eight homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ mice (● −/−) eight homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ mice with the *Del7C* transgene (line 1 −/− ■) and eight homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ mice with the *Del7C* transgene (line 2 −/− ▲), 12 normal controls (▼ +/+) and 15 homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ mice with the normal transgene (−/− ◆ +). Each vertical line represents the standard error of the mean.

lower than the PDE activity measured for the 12-day-old +/+ control retina which is inhibited by PDEγ (Hurley & Stryer, 1982; Stryer, 1991). The discrepancy between in vitro and in vivo results can be explained by the observed reduction in the expression of the catalytic

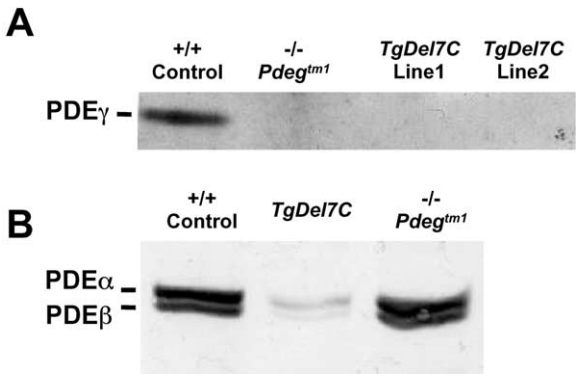

Fig. 4. Immunoblot analysis of the expression of PDE subunits in control, Tg*Del7C* and −/− $Pdeg^{tm1}$ retinal homogenates, normalized for protein. (A) Immunoblot incubated with a polyclonal antibody recognizing the *N*-terminal of the PDEγ subunit. Samples loaded at 10 μg/lane. Lane 1, +/+ control; lane 2, −/− $Pdeg^{tm1}/Pdeg^{tm1}$; lanes 3 and 4, transgenic *Del7C* (lines 1 and 2, respectively). (B) Immunoblot incubated with a polyclonal peptide antibody recognizing both the PDEα and β subunits. Samples loaded at 10 μg/lane. Lane 1, +/+ control; lane 2, Tg*Del7C*; lane 3, −/− $Pdeg^{tm1}/Pdeg^{tm1}$.

PDEα and β subunits in the Tg*Del7C* animals (Fig. 4B), which indirectly suggests a high basal specific activity of the Tg*Del7C* PDE.

Furthermore, animals expressing the *Del7C* mutant allele did not exhibit a substantial photoresponse as judged by the ERG measurements (Figs. 2 and 3). However, the amplitude of the response of Tg*Del7C* mutants was larger and the response was more sensitive than that of the $Pdeg^{tm1}/Pdeg^{tm1}$ mice. The retinal function of Tg*Del7C* mutants decayed gradually over time similar to what happens in individuals with retinitis pigmentosa. Retinal degeneration in the *Del7C* may be due to inability of the truncated PDEγ transgene product to support sufficient expression of the PDE catalytic core function (Tsang et al., 1996). This suggests that a novel positive role of PDEγ in promoting PDE activity (Tsang et al., 1996) could be found within its carboxyl tail. Thus, the PDEγ subunit carboxyl terminus is deemed essential to maintain the proper function and integrity of the photoreceptors.

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